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APPLICATION NUMBER: *60/422,712*
FILING DATE: *October 30, 2002*
RELATED PCT APPLICATION NUMBER: *PCT/US03/34837*

By Authority of the
COMMISSIONER OF PATENTS AND TRADEMARKS

L. wallace

T. WALLACE
Certifying Officer

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PTO/SB/16 (8-00)
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JC601 U.S. PTO 10/30/02

JC050 U.S. PTO 60/422712

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Ralf	Koehler	Berlin, Germany
Heike	Wulff	Irvine, California
Joachim	Hoyer	Berlin, Germany

☒ *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (280 characters max)

NON-PEPTIDE INHIBITION OF VASCULAR ENDOTHELIAL PROLIFERATION AND THERAPIES RELATED THERETO

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☒ Specification *Number of Pages* 8
☐ Drawing(s) *Number of Sheets*
☒ Application Data Sheet. See 37 CFR 1.76
☐ CD(s), Number
☒ Other (specify) Appendix A(incl. Pages 1-26 of written text & Figures 1-5);Postcard

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☒ Applicant claims small entity status. See 37 CFR 1.27.
☐ A check or money order is enclosed to cover the filing fees
☒ The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number 50-0878
☐ Payment by credit card. Form PTO-2038 is attached.

FILING FEE AMOUNT ($) $80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☒ No.
☐ Yes, the name of the U.S. Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE

Date 10/30/02

TYPED or PRINTED NAME Robert D. Buyan

REGISTRATION NO. *(if appropriate)* 32,460

Docket Number: UCIVN

TELEPHONE 949-450-1750

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C.

P19SMALL/REV05

PROVISIONAL APPLICATION COVER SHEET
Additional Page

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Docket Number	UCIVN	Type a plus sign (+) inside this box →	+

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K. George	Chandy	Laguna Beach, California
Michael D.	Cahalan	Newport Coast, California

Number 2 of 2

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Attorney Docket No. UCIVN-020N

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Ralf Koehler, et al.

Serial No.: To Be Determined

Filed: Herewith, October 30, 2002

Title: Non-Peptide Inhibition of Vascular Endothelial Proliferation and Therapies Related Thereto

Transmittal of Provisional Application for Patent
37 CFR 1.53 (b) (2)

Express Mail Mailing Label No. EV097454885US

Box Provisional Application
Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Enclosed, for filing in the United States Patent Office under 37 CFR 1.53 (b)(2), please find the following documents:

1. Provisional Patent Application entitled ""Non-Peptide Inhibition of Vascular Endothelial Proliferation and Therapies Related Thereto" consisting of 8 total pages plus Appendix A (Appendix A consists of Pages 1-26 of written text and accompanying Figures 1-5)
2. A completed Provisional Application Cover Sheet consisting of 1 page;
3. Application Data Sheet;
4. Check No. 2812 in the amount of $80.00; and
5. A Return Postcard

The inventors of the invention(s) disclosed in this Provisional Patent Application are:

Ralf Koehler
Heike Wulff
Joachim Hoyer
K. George Chandy
and
Michael D. Cahalan

The Notice to File Missing Parts (Filing Date Granted) should be mailed to applicant's undersigned counsel at the address shown here below.

Respectfully submitted,

STOUT, UXA, BUYAN & MULLINS, LLP

Date: October 30, 2002

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CERTIFICATE OF MAILING

I hereby certify that this transmittal letter and the accompanying Provisional Patent Application entitled "Non-Peptide Inhibition of Vascular Endothelial Proliferation and Therapies Related Thereto" are being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR § 1.10 on October 30, 2002 and is addressed to Box Provisional Application, Commissioner for Patents, Washington, D.C. 20231.

Date: October 30, 2002

By: Francine Sanders, Assistant

APPLICATION DATA SHEET

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Application Information

Title Line One::	Non-Peptide Inhibition of Vascular Endothelial
Title Line Two::	Proliferation and Therapies Related Thereto
Total Drawing Sheets::	0
Formal Drawings?::	No
Application Type::	Provisional
Docket Number::	UCIVN-020N

Representative Information

Registration Number One::	34,493
Registration Number Two::	25,612
Registration Number Three::	32,460

Registration Number Four::	36,331
Registration Number Five::	42,243
Registration Number Six::	43,215
Registration Number Seven::	38,883
Registration Number Eight::	45,526
Registration Number Nine:	45,374
Registration Number Ten:	32,337

Continuity Information

This application is a::	N/A
>Application One::	N/A
Filing Date::	
>Application Two::	
Filing Date::	

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PROVISIONAL APPLICATION FOR UNITED STATES PATENT

by

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Heike Wulff

Joachim Hoyer

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and

Michael D. Cahalan

assignors to

The Regents of The University of California

for

NON-PEPTIDE INHIBITION OF VASCULAR ENDOTHELIAL PROLIFERATION AND THERAPIES RELATED THERETO

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DOCKET NO. UCIVN-020N

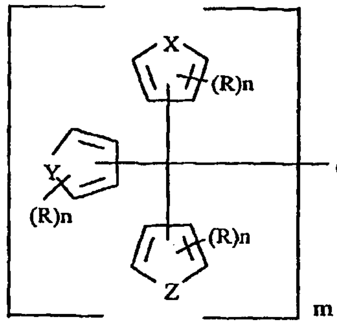
Express Mail No. EV097454885US

NON-PEPTIDE INHIBITION OF VASCULAR ENDOTHELIAL PROLIFERATION AND THERAPIES RELATED THERETO

The present invention provides compositions, preparations and methods for treating or preventing vascular stenosis or proliferation of vascular endothelium in human or veterinary patients, as may occur in various diseases and disorders such as athersclerosis and diseases of arteries, other blood vessels and/or bypass grafts, restenosis of blood vessels and/or bypass grafts following treatment procedures (e.g., restenosis following balloon angioplasty, atherectomy, in-stent restenosis, neointimal thickening, etc).

Ca^{2+}-activated K^+ channels (K_{Ca}) are important regulators of vascular smooth muscle function. The intermediate-conductance K_{Ca} channel encoded by the IKCa1 gene (a.k.a IK1, hSK4, KCa4 and $K_{Ca}3.1$ as per the new IUPHAR nomenclature: http://www.iuphar.org/compendium2.htm) has been proposed to be an important regulator of cell proliferation. In human lymphocytes and fibroblasts, an up-regulation of IKCa1 expression has been shown to be an essential step in promoting cell proliferation. The present invention includes the inhibition of the intermediate-conductance K_{Ca} channel encoded by the IKCa1 gene to treat, prevent or reverse vascular smooth muscle cell proliferation and/or conditions that result in whole or in part from vascular smoth muscle cell proliferation, such as atherosclerosis, vascular stenosis, vascular restenosis, etc.

In accordance with the present invention, TRAM 34 and/or related compounds and/or pharmaceutically acceptable salts or derivatives thereof and/or any other compounds which block or inhibit Ca^{2+}-activated K^+ channels (K_{Ca}) (e.g., are administered to human or veterinary patients via route(s) of administration and in dosages that are effective to deter, inhibit, prevent or reverse vascular proliferation. TRAM 34 and related compounds as disclosed herein are specifically described in PCT Internationbal Publication No. WO 01/49663, the entirety of which is expressly incorporated herein by reference. In general, these compounds have Structural Formula I as follows:



Formula I

Wherein,

X,Y and Z are same or different and are independently selected from CH2, O, S, NR_1, N=CH, CH=N and R_2-C=C-R_3, where R_2 and R_3 are H or may combine to form a saturated or unsaturated carbocyclic or heterocyclic ring, optionally substituted with one or more R groups;

R_1 is selected from H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, acyl and aroyl, optionally substituted with hydroxy, amino, substituted amino, cyano, alkoxy, halogen, trihaloalkyl, nitro, thio, alkylthio, carboxy and alkoxycarbonyl groups;

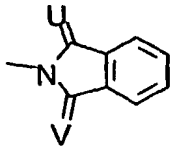
R is selected from H, halogen, trihaloalkyl, hydroxy, acyloxy, alkoxy, alkenyloxy, thio, alkylthio, nitro, cyano, ureido, acyl, carboxy, alkoxycarbonyl, N-(R_4)(R_5) and saturated or unsaturated, chiral or achiral, cyclic or acyclic, straight or branched hydrocarbyl group with from 1 to 20 carbon atoms, optionally substituted with hydroxy, halogen, trihaloalkyl, alkylthio, alkoxy, carboxy, alkoxycarbonyl, oxoalkyl, cyano and N-(R_4)(R_5) group,

R_4 and R_5 are selected from H, alkyl, alkenyl, alkynyl, cycloalkyl and acyl or R_4 and R_5 may combine to form a ring, wherein a carbon may be optionally substituted by a heteroatom selected from O, S or N-R_6,

R_6 is H, alkyl, alkenyl, alkynyl, cycloalkyl, hydroxyalkyl or carboxyalkyl,

n is 1-5; m is 1 or 2; with the proviso that when m is 1, Q is selected from OH, CN,

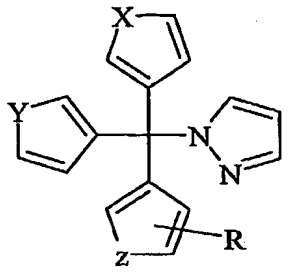
carboxyalkyl, N-(R_7)(R_8), where R_7 and R_8 are selected from H, lower alkyl (1-4C), cycloalkyl, aryl, acyl, amido, or R_7 and R_8 may combine to form a saturated or unsaturated heterocylic ring and optionally substituted with up to 3 additional heteroatoms selected from N, O, and S; or
-NH-heterocycle, where the heterocycle is represented by thiazole, oxazole, isoxazole, pyridine, pyrimidine, and purine and
where U and V are selected from H and O; and



when m is 2, Q is a spacer of from 2-10 carbons as a straight or branched, chiral or achiral, cyclic or acyclic, saturated or unsaturated, hydrocarbon group, such as phenyl.
In the most preferred embodiment of this invention,
X, Y, and Z are R_2-C=C-R_3, where R_2 and R_3 are H;
R is selected from H and halogen, preferably, F and Cl;
m is 1; and
Q is -N-(R_7)(R_8), where R_7 and R_8 are selected from H, acyl, amido, and R_7 and R_8 combine to form a saturated or unsaturated heterocyclic ring, optionally substituted with up to three heteroatoms selected from N, O, or S, for example, pyrrolidine, piperidine, pyrazole, imidazole, oxazole, isoxazole, tetrazole, azepine, etc., which may be optionally substituted with a lower alkyl or amino group.

Compounds of Formula I have been determined to selectively inhibit the intermediate-conductance calcium-activated potassium channel, *IKCa1*, at low nanomolar concentrations, and exhibit 200-1500 fold selectivity for this channel over other ion channels.

Further in accordance with the invention, preferred compounds of this invention having the general Formula I above, are a group of triarylmethyl-1*H*-pyrazole compounds that have structural Formula I-A below:



FORMULA I-A

Wherein:

X, Y, and Z are R_2-C=C-R_3, where R_2 and R_3 are H;
R is selected from H and halogen, preferably, F and Cl;

Still further in accordance with the invention, 1-[(2-chlorophenyl)diphenyl methyl]-1*H*-pyrazole (designated as TRAM-34) and possibly other compounds of Formulas I and I-A above, when administered to human and/or veterinary patients, inhibit or prevent or reverse neointimal thickening or proliferation of the vascular endothelium of the patient's arteries.

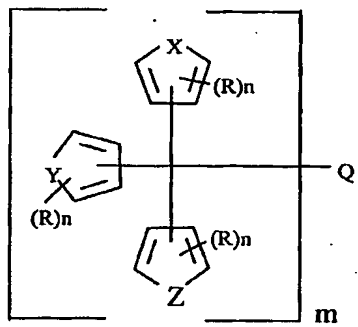
Further disclosure of this invention is set forth in the manuscript appended hereto as Exhibit A entitled "Blockade of the IKCa1 Ca^{2+}-activated K^+ channel as a new therapeutic strategy for restenosis" (Pages 1-26 of written text and accompanying Figures 1-5), the entirety of which is included in and forms a part of this provisional patent application.

DISCLOSURE STATEMENTS IN CLAIM FORMAT

The present invention, as disclosed in the provisional patent application includes but is not necessarily limited to the following:

1. A method for inhibiting proliferation of vascular smooth muscle cells and/or for treating, preventing or reversing stenosis or restenosis of a blood vessel in a human or veterinary patient, said method comprising the step of:

administering to the patient a therapeutically effective amount of a compound having the structural formula



Wherein,

X,Y and Z are same or different and are independently selected from CH2, O, S, NR_1, N=CH, CH=N and R_2-C=C-R_3, where R_2 and R_3 are H or may combine to form a saturated or unsaturated carbocyclic or heterocyclic ring, optionally substituted with one or more R groups;

R_1 is selected from H, alkyl, alkenyl, alkynyl, cycloalkyl, aryl, acyl and aroyl, optionally substituted with hydroxy, amino, substituted amino, cyano, alkoxy, halogen, trihaloalkyl, nitro, thio, alkylthio, carboxy and alkoxycarbonyl groups;

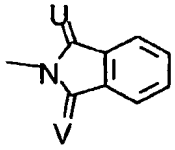
R is selected from H, halogen, trihaloalkyl, hydroxy, acyloxy, alkoxy, alkenyloxy, thio, alkylthio, nitro, cyano, ureido, acyl, carboxy, alkoxycarbonyl, N-(R_4)(R_5) and saturated or unsaturated, chiral or achiral, cyclic or acyclic, straight or branched

hydrocarbyl group with from 1 to 20 carbon atoms, optionally substituted with hydroxy, halogen, trihaloalkyl, alkylthio, alkoxy, carboxy, alkoxycarbonyl, oxoalkyl, cyano and N-(R_4)(R_5) group,

R_4 and R_5 are selected from H, alkyl, alkenyl, alkynyl, cycloalkyl and acyl or R_4 and R_5 may combine to form a ring, wherein a carbon may be optionally substituted by a heteroatom selected from O, S or N-R_6,

R_6 is H, alkyl, alkenyl, alkynyl, cycloalkyl, hydroxyalkyl or carboxyalkyl,

n is 1-5; m is 1 or 2; with the proviso that
when m is 1, Q is selected from OH, CN, carboxyalkyl, N-(R_7)(R_8), where R_7 and R_8 are selected from H, lower alkyl (1-4C), cycloalkyl, aryl, acyl, amido, or R_7 and R_8 may combine to form a saturated or unsaturated heterocylic ring and optionally substituted with up to 3 additional heteroatoms selected from N, O, and S; or
-NH-heterocycle, where the heterocycle is represented by thiazole, oxazole, isoxazole, pyridine, pyrimidine, and purine and
where U and V are selected from H and O; and



when m is 2, Q is a spacer of from 2-10 carbons as a straight or branched, chiral or achiral, cyclic or acyclic, saturated or unsaturated, hydrocarbon group, such as phenyl.
In the most preferred embodiment of this invention,
X, Y, and Z are R_2-C=C-R_3, where R_2 and R_3 are H;
R is selected from H and halogen, preferably, F and Cl;
m is 1; and
Q is -N-(R_7)(R_8), where R_7 and R_8 are selected from H, acyl, amido, and R_7 and R_8 combine to form a saturated or unsaturated heterocyclic ring, optionally substituted with up to three heteroatoms selected from N, O, or S, for example, pyrrolidine, piperidine, pyrazole, imidazole, oxazole, isoxazole, tetrazole,

azepine, etc., which may be optionally substituted with a lower alkyl or amino group.

2. A method according to Claim 1 wherein the X, Y, and Z are each R_2-C=C-R_3 (where R_2 and R_3 are H; R is selected from H and halogen, preferably, F and Cl); m is 2; and Q is a spacer of from 2-10 carbons either as a straight or branched hydrocarbon chain, or containing a hydrocarbon ring.

3. A method according to Claim 1 wherein the compound is 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole.

4. A method according to Claim 1 wherein the compound is 1-[(2-fluorphenyl)diphenylmethyl]-1*H*-pyrazole .

5. A method according to Claim 1 wherein the compound is 1-[(4-chlorophenyl)diphenylmethyl]-1*H*-pyrazole .

6. A method according to Claim 1 wherein the compound is 1-[(2-fluorphenyl)diphenylmethyl]-1*H*-pyrazole .

7. A method according to Claim 1 wherein the compound is 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-1,2,3,4-tetrazole.

8. The use of TRAM 34 and/or any compound of Structural Formula I or IA, or any compound recited in Nos. 1-7 above, for the treatment, prevention or reversal of atherosclerosis and diseases of arteries, other blood vessels and/or bypass grafts, restenosis of blood vessels and/or bypass grafts following treatment procedures (e.g., restenosis following balloon angioplasty, atherectomy, in-stent restenosis, neointimal thickening, etc.

9. The use of TRAM 34 and/or any compound of Structural Formula I or IA, or any compound recited in Nos. 1-7 above, in the manufacture of a

preparation for the treatment, prevention or reversal of atherosclerosis and diseases of arteries, other blood vessels and/or bypass grafts, restenosis of blood vessels and/or bypass grafts following treatment procedures (e.g., restenosis following balloon angioplasty, atherectomy, in-stent restenosis, neointimal thickening, etc.

10. A method for inhibiting proliferation of vascular smooth muscle cells and/or for treating, preventing or reversing stenosis or restenosis of a blood vessel in a human or veterinary patient, said method comprising the step of:

(a) inhibiting flux through or blocking Ca^{2+}-activated K^+ channels (K_{Ca}) so as to inhibit vascular smoth muscle cell proliferation.

11. A method according to No. 10 above wherein Step A comprises inhibiting or blocking the IKCal Ca^{2+}-activated K^+ channel.

12. A method according to No. 10 or 11 above wherein Step A comprises administering to the patient a therapeutically effective amount of TRAM 34 or a compound of General Formula I or IA or any compound recited in any of Nos. 1-7 above or any other compound inhibits flux through or blocks the IKCal Ca^{2+}-activated K^+ channel or any other Ca^{2+}-activated K^+ channel such that proliferation of vascular smoth muscle cells is inhibited.

13.

Fig 1

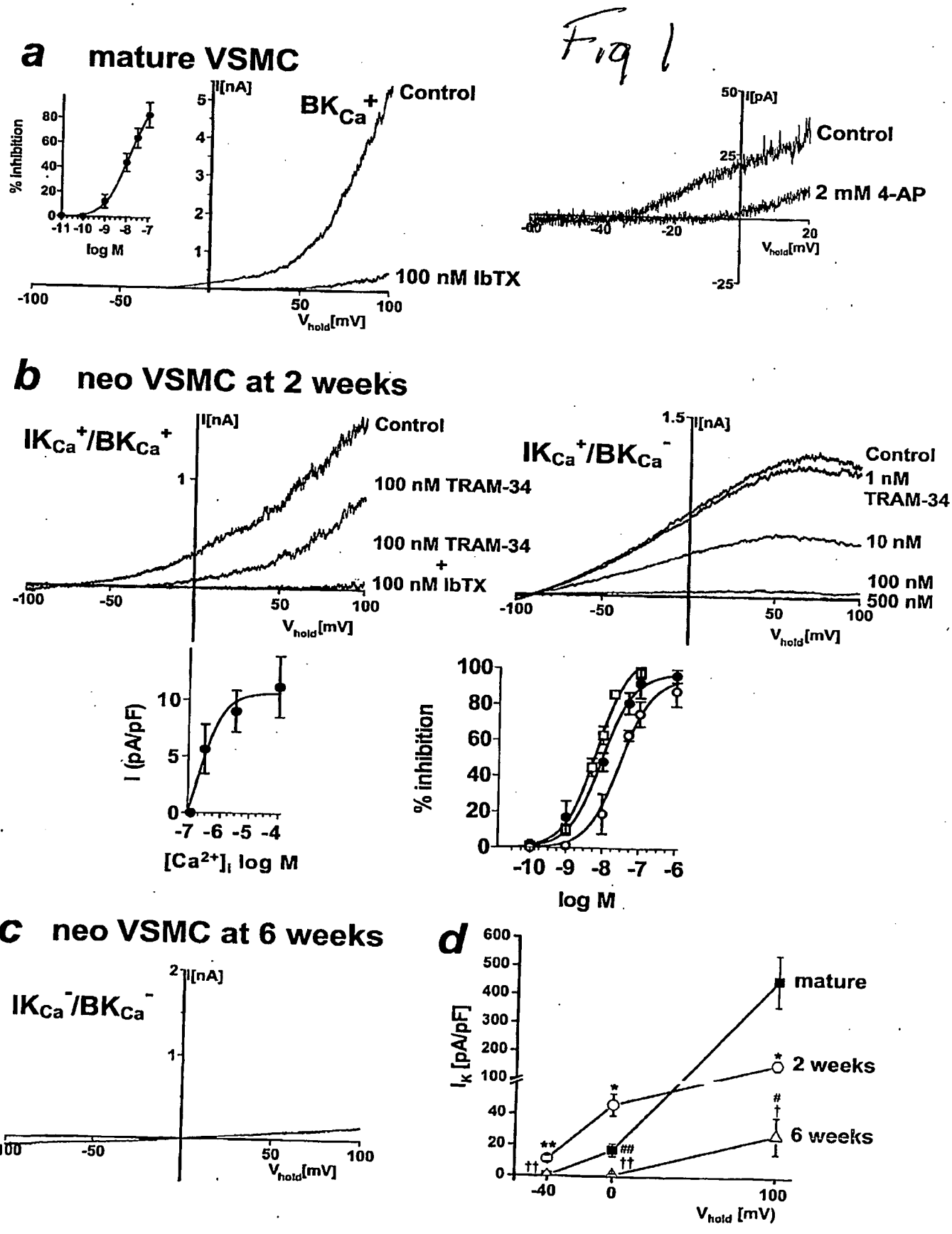
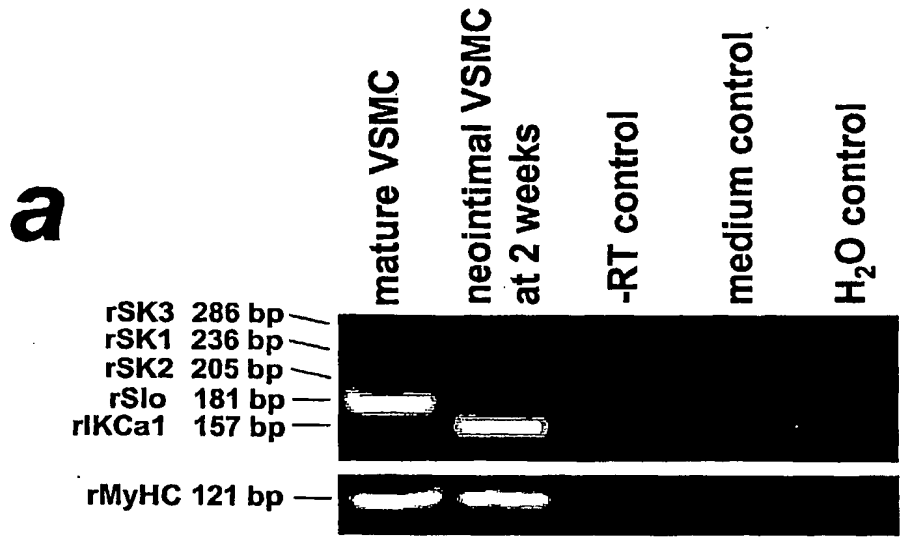


Fig 2



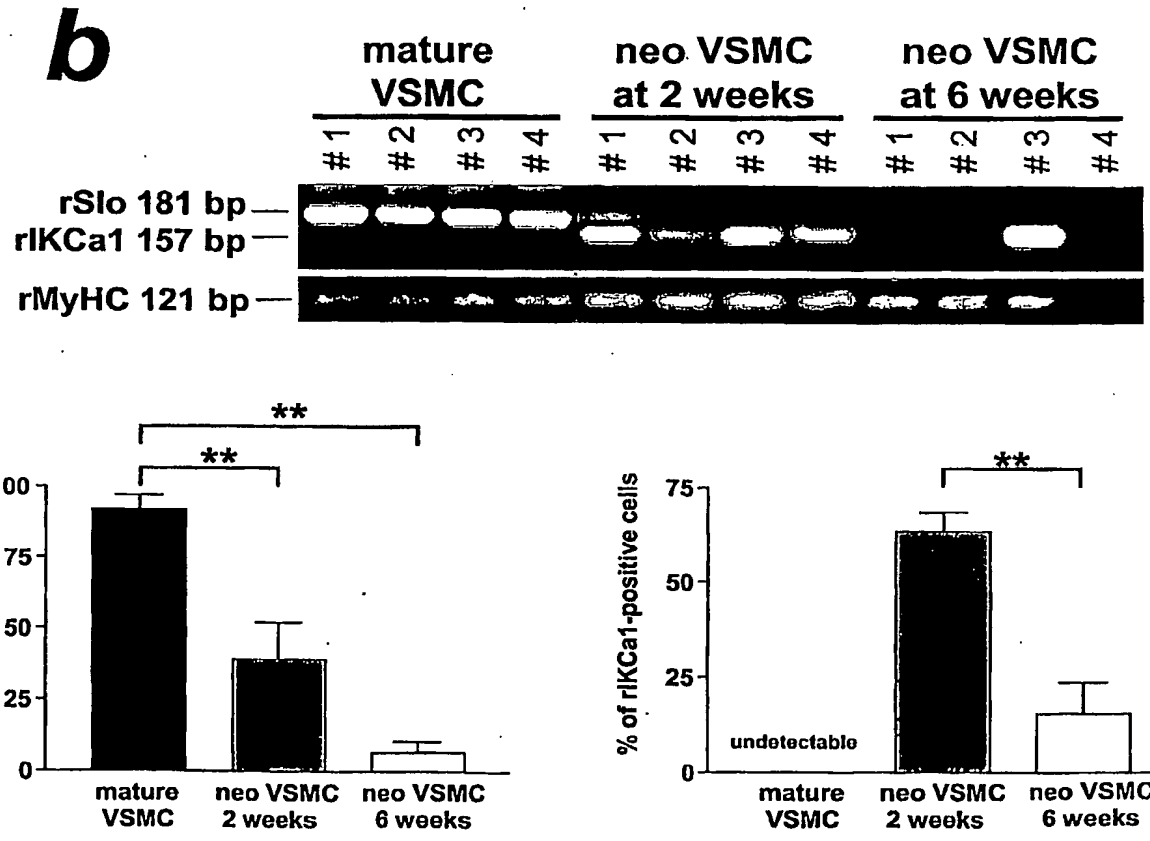
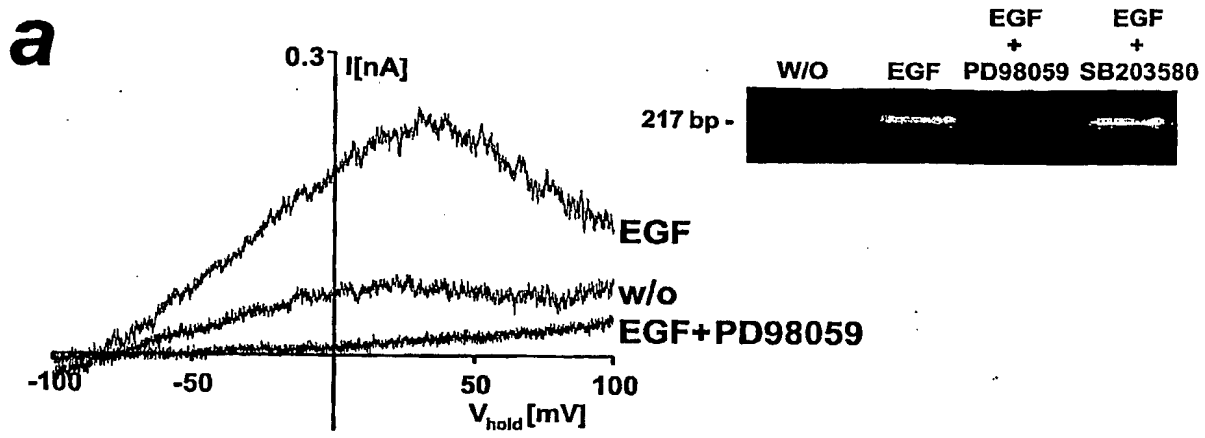


Figure 2



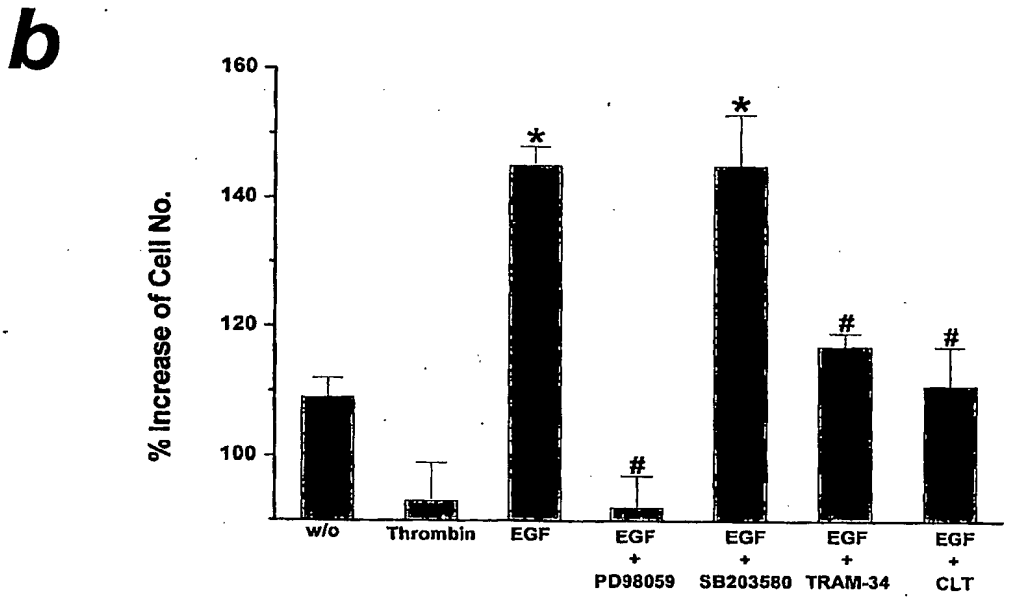


Fig 3

Figure 3

Fig. 4

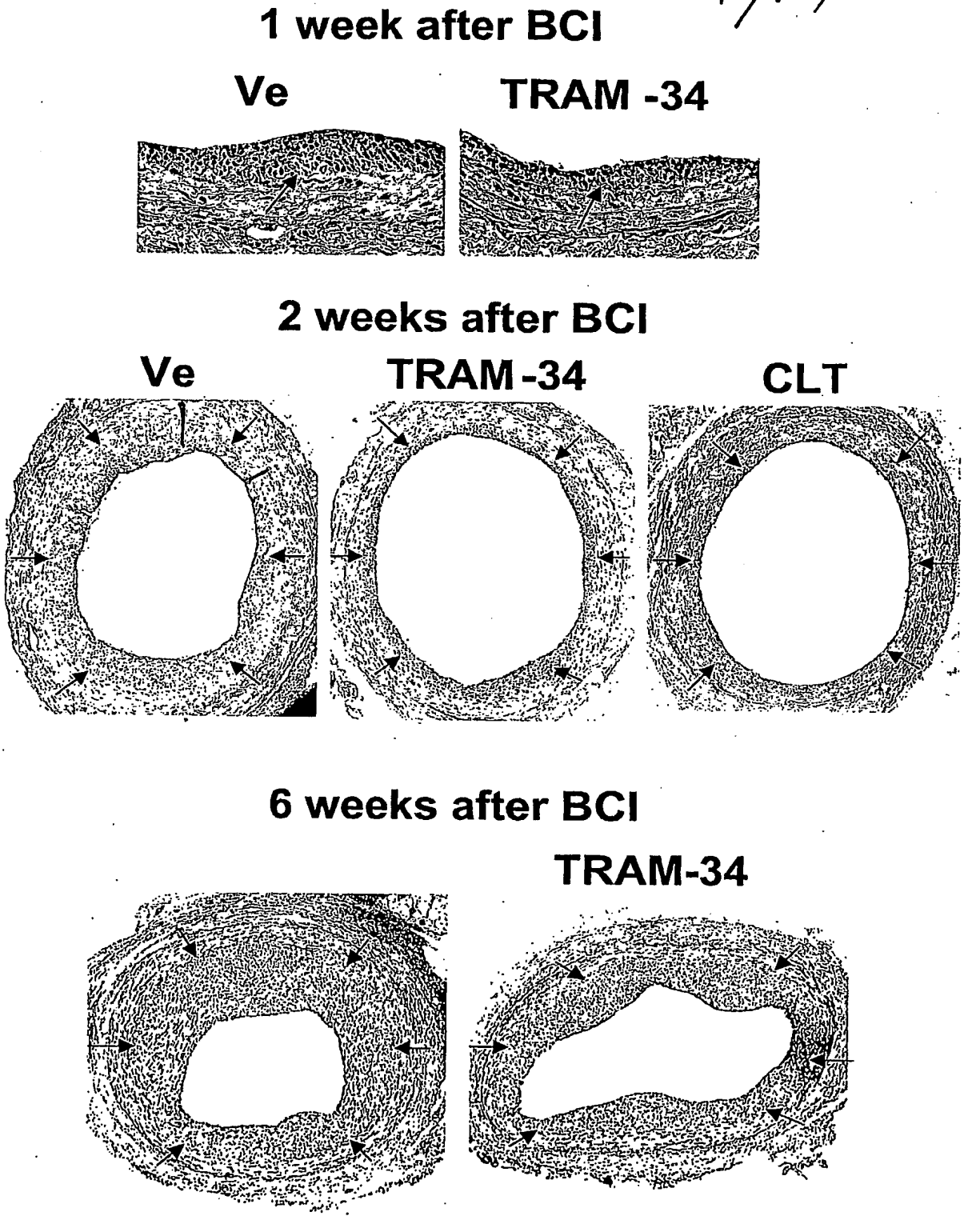


Figure 4

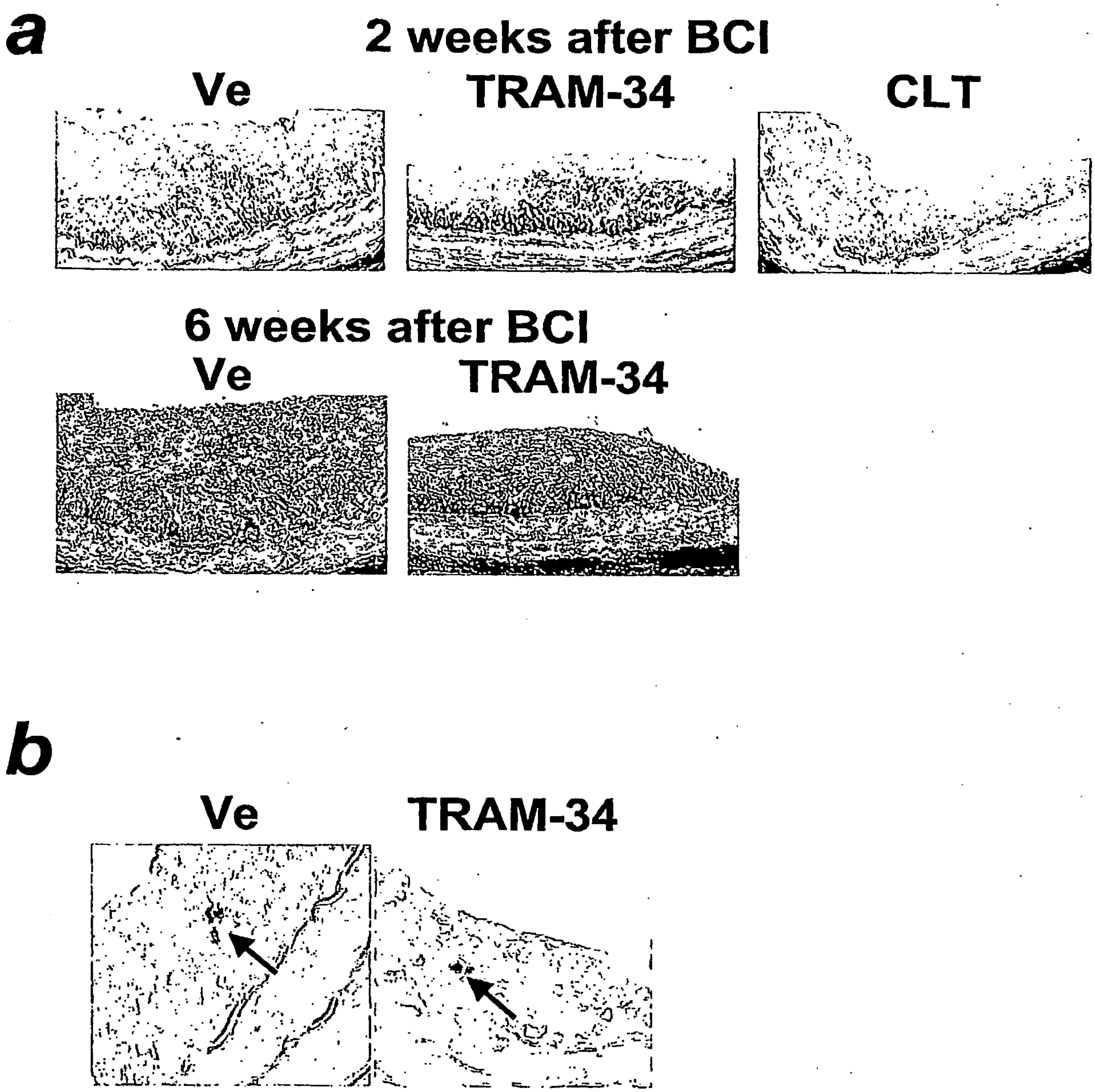


Fig 5

Figure 5

Blockade of the IKCa1 Ca^{2+}-activated K^+ channel as a new therapeutic strategy for restenosis.

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Nonstandard Abbreviations used: apamin (APA); balloon catheter injury (BCI); carotid artery (CA); clotrimazole (CLT); intermediate-conductance Ca^{2+}-activated potassium channel (IK_{Ca}); 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole (TRAM-34).

Abstract

Angioplasty stimulates proliferation and migration of vascular smooth muscle cells (VSMC) leading to neointimal thickening and vascular restenosis. In a rat model of balloon catheter injury (BCI) we investigated whether alterations in expression of Ca^{2+} activated K^+ channels (K_{Ca}) are involved in intimal hyperplasia. Mature medial VSMC exclusively expressed large-conductance K_{Ca} (BK_{Ca}) channels. Two weeks after BCI, expression of BK_{Ca} was significantly reduced in neointimal VSMC, while expression of intermediate-conductance K_{Ca} (IKCa1) channels was significantly augmented. Using the aortic VSMC cell line A7r5 we ascertained that IKCa1 up-regulation occurred via epidermal growth factor (EGF)-mediated activation of the MEK/ERK pathway. EGF-induced cell VSMC proliferation *in vitro* was suppressed by the selective IKCa1 blocker TRAM-34, and daily *in vivo* administration of TRAM-34 (120 mg/kg) to rats significantly reduced intimal hyperplasia by ~40% at one, two, and six weeks after BCI. Two weeks treatment with the parent compound clotrimazole (120 mg/kg/d) was equally effective. Reduction of intimal hyperplasia was accompanied by decreased neointimal cell content with no change in the rate of apoptosis or collagen content. The switch towards IKCa1 expression may promote excessive neointimal VSMC proliferation and blockade of IKCa1 could therefore represent a new therapeutic strategy to prevent restenosis after angioplasty.

Key words: angioplasty - restenosis - TRAM-34 - clotrimazole

Introduction

Restenosis is a major complication after percutaneous balloon angioplasty. This intervention to relieve arterial stenosis and to improve blood flow, initiates proliferation of VSMC leading to a substantial re-narrowing of the vessel lumen or complete restenosis within weeks (1). Complex interactions between numerous growth-stimulating molecules have been proposed to promote migration and proliferation of VSMC (2) and thus lead to neointima formation. Proliferating VSMC are characterized by alterations in functional plasticity as they switch from a contractile phenotype to a de-differentiated phenotype.

Ca^{2+}-activated K^+ channels (K_{Ca}) are important regulators of VSMC function (3,4). Mature VSMC predominantly express the voltage-dependent and calcium-activated large-conductance channel (BK_{Ca} or maxi K) (4), a product of the *Slo* gene (5), which plays a pivotal role in VSMC relaxation and vasodilation, its hyperpolarizing action prohibiting depolarization-dependent activation of Ca^{2+} channels and subsequent Ca^{2+} influx (3,4). In contrast to the vasodilatory function of BK_{Ca}, the role of other channels of the K_{Ca} gene family in VSMC is incompletely understood. The intermediate-conductance K_{Ca} channel encoded by the IKCa1 gene (a.k.a IK1, hSK4, KCa4 and $K_{Ca}3.1$ as per the new IUPHAR nomenclature: http://www.iuphar.org/compendium2.htm) has been proposed to be an important regulator of cell proliferation. In human lymphocytes and fibroblasts, an up-regulation of IKCa1 expression has been shown to be an essential step in promoting cell proliferation (6,7,8).

In this study we tested the hypothesis that a reorganization of K_{Ca} channel expression pattern after angioplasty promotes neointimal cell proliferation. Following BCI to the carotid artery of rats, we found that neointimal VSMC switch K_{Ca} gene expression from *Slo* to *IKCa1*, representing a change from a K_{Ca} subtype mediating vasodilation to a K_{Ca} subtype promoting cell proliferation. The molecular mechanism underlying this augmentation of IKCa1 expression

involved EGF-induced activation of MEK/ERK pathway. Blockade of IKCa1 by the antimycotic clotrimazole (CLT) and its more selective-derivative TRAM-34 (6) resulted in inhibition of EGF-stimulated VSMC proliferation *in vitro* and in a significant reduction in neointima formation *in vivo* following BCI.

Methods

Animals. Three to four month-old male Sprague-Dawley rats (350-450g) were purchased from the Animal Breeding Center Schönewalde GmbH (Schönewalde, Germany).

Cell line. Commercially available rat aortic VSMC (A7r5) were cultured in DMEM containing 1 mM sodium pyruvate, non-essential amino acids, penicillin, (20 units/ml), streptomycin (20 μg/ml), and 10% fetal calf serum (all Biochrom KG, Berlin, Germany).

Reagents. PD98059 and SB203580 were obtained from TOCRIS (Ballwin, MO). TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole) was synthesized as described previously (*6*); TRAM-34 was dissolved in dimethyl sulfoxide for *in vitro* assays and in peanut oil for *in vivo* administration. sEGF was obtained from Biochrom KG Berlin, Germany. All other chemicals and toxins were obtained from Sigma (Deisenhofen, Germany).

Balloon catheter injury and treatment protocols. Under the aegis of an animal study protocol approved by the Animal Care and Use Committee of the Freie Universität, Berlin, rats were subjected to BCI of the left carotid artery (CA) by use of a 2F Fogarty embolectomy catheter (Baxter Scientific, Irvine, CA) (9). Rats were sacrificed two weeks (n = 5) and six weeks (n = 6) after BCI, and left and right CA were excised. Separate groups of rats (each n = 4-11) were treated with daily subcutaneous injections of TRAM-34 (120 mg/kg) or the vehicle (peanut oil) for one, two, and six weeks after BCI. Another group (n = 7) was treated with CLT (120 mg/kg) for two weeks after BCI. TRAM-34 and CLT serum levels were quantitatively determined by a bioassay as described previously (10).

Neointimal thickening was determined at one, two, and six weeks after BCI in paraffin embedded and differential non-serial cross sections stained with hematoxylin and eosin to visualize nuclei and cytoplasm, or with Sirius Red to detect collagen. Cross sectional areas of the neointimal and medial smooth-muscle-cell layers, the neointima/media ratio, and collagen content were calculated with a computerized analysis system (Scion Image, Scion Corporation, Frederick, Maryland). Analysis was done in a blinded manner.

Patch-clamp experiments. All experiments were conducted in the whole-cell configuration of the patch-clamp technique and data analysis was performed as described (12,13). If not otherwise stated, cells were dialyzed with a pipette solution containing (mM): 135 KCl, 4 $MgCl_2$, 1 EGTA, 0.955 $CaCl_2$, ($[Ca^{2+}]_{free}$ = 3 μM), and 5 HEPES (pH 7.2). For determination of Ca^{2+}-dependence of K_{Ca} channels, cells were dialyzed with pipette solutions containing different $[Ca^{2+}]_{free}$ concentrations and the average current density was calculated for each $[Ca^{2+}]$. The NaCl bath solution contained (mM): 137 NaCl, 4.5 Na_2HPO_4, 3 KCl, 1.5 KH_2PO_4, 0.4 $MgCl_2$, and 0.7 $CaCl_2$ (pH 7.4).

Detection of apoptosis. Apoptotic nuclei in the neointima were detected by the terminal transferase-mediated fluorescein-conjugated dUTP nick end labeling (TUNEL) method (Apoptaq® Plus; Qbiogene, Heidelberg, Germany) according to the manufacturer's instructions. Slices were counterstained with methyl green to visualize all nuclei.

In vitro proliferation studies. To induce growth arrest, A7r5 cells were kept in serum-free medium for 48h prior to stimulation with EGF (20 ng/ml) or thrombin (1 U/ml) with or without TRAM-34 (1 μM), CLT (1 μM), PD98059 (20 μM), or SB203580 (5 μM). At 5-10% confluence,

photomicrographs of cells were taken in a fixed field before and 48h after stimulation. Cells were counted and the % increase in cell count was calculated for each experiment.

RNA Isolation and quantitative realtime RT-PCR. Cells were harvested at 2h or 48h after stimulation by scrapping. RNA was isolated and purified using TRIZOL (Life Technologies, Eggenstein, Germany), following the manufacturer's instructions. RNA (2 µg) was reverse transcribed using random hexamers (Boehringer, Mannheim, Germany) and M-MLV reverse transcriptase (Life Technologies, Eggenstein, Germany) in a 50 µl reaction. Expression was quantified with an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems Inc). Primers were positioned in the coding region and spanned intronic sequences. Internal oligonucleotides (Biotez, Berlin, Germany) were labeled with 6-carboxy-fluorescein (FAM) on the 5' end and 6-carboxytetramethylrhodamine (TAMRA) on the 3' end. Identity of PCR products was verified by sequencing and linearity of each PCR assay were confirmed by serial dilutions of cDNA. Primer pairs and internal oligonucleotides:

rIKCa1: F 5`-CTGAGAGGCAGGCTGTCAATG-3′; R 5`-ACGTGTTTCTCCGCCTTGTT-3′; P 5`-AAGATTGTCTGCTTGTGCACCGGAGTC-3′;

rat myosin heavy chain (rMyHC): F 5′-CATCAATGCCAACCGCAG-3′; R 5′-TCCCGAGCATCCATTTCTTC-3′; P 5′-TGAGGCCATGGGCCGTGAGG-3′;

rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH): F 5′-CGGCACAGTCAAGGCTGAG-3′; R 5′-CAGCATCACCCCATTTGATGT-3′; P 5′-CCCATCACCATCTTCCAGGAGCGA-3′.

Each 25 µl PCR reaction consisted of 500 nM forward primer, 500 nM reverse primer, 150 nM probe, 3 µl cDNA, and 1x (final concentration) TaqMan Universal Master Mix (Perkin-Elmer Applied Biosystems Inc). PCR parameters were 50°C x 2min, 95°C x 10 min, and 50 cycles at 95°C x 15 s, 60°C x 1 min.

The TaqMan® software was employed to calculate a threshold cycle (Ct) which is defined as the cycle at which the reporter fluorescence is distinguishable from the background in the extension phase of the PCR reaction (ABI User Bulletin #2). Real-time RT-PCR signals for rIKCa1 and rMyHC were standardized to rGAPDH by using the equation: $Ct_X - Ct_{rGAPDH} = \Delta Ct$, where Ct_X is the value for the rIKCa1 or the rMyHC probe, and Ct_{rGAPDH} is the value calculated for rGAPDH. The equation, $\Delta Ct_{w/o} - \Delta Ct_X = \Delta\Delta Ct$, was used to determine changes in expression following EGF stimulation, where the experimental ΔCt_X value was subtracted from the control $\Delta Ct_{w/o}$ value (w/o = without stimulus) of the same experiment. Fold increases in expression were calculated by the equation, $2^{\Delta\Delta Ct}$ = fold change in expression (ABI User Bulletin #2).

In situ cell harvesting and reverse transcription. In situ harvesting of single neointimal VSMC from freshly isolated CA segments, isolation of mature VSMC from healthy CA, reverse transcription of mRNA from single cell samples, and "multiplex" single cell RT-PCR were performed as described previously (12). First and 'nested' primer pairs spanning intronic sequences for rSlo, small K_{Ca} (rSK1-3), and rIKCa1 were used for the K_{Ca} channels. Primers for rMyHC and endothelial nitric oxide synthase (reNOS) served as markers for VSMC and endothelial cells. Identity of PCR products was verified by sequencing. Forward and reverse primer:

rIKCa1: first: 5′-GAGAGGCAGGCTGTCAATG-3′; 5′-GGGAGTCCTTCCTTCGAGTG-3′; nested: 5′-CATCACGTTCCTGACCATTG-3′; 5′-GTGTTTCTCCGCCTTGTTGA-3′;

rSlo: first: 5′-GGACTTAGGGGATGGTGGTT-3′; 5′-GGGATGGAGTGGACAGAGGA-3′; nested: 5′-TTTACCGGCTGAGAGATGCC-3′; 5′-TGTGAGGAGTGGGAGGAATGA-3′;

rSK1: first: 5′-GCACACCTACTGTGGGAAGG-3′; 5′-AGCTCCGACACCACCTCATA-3′; nested: 5′-GCTGAGAAACACGTGCACAA-3′; 5′-TTGGCCTGATCATTCACCTT-3′;

rSK2: first: 5′-GGAATAATGGGTGCAGGTTG-3′; 5′-TTTGTTTCCAGGGTGACGAT-3′; nested: 5′-CTTGGTGGTAGCCGTAGTGG-3′; 5′-GAATTTCCGTTGATGCTTCC-3′;

rSK3: first: 5′-AACCCCTCCAGCTCTTCAGT-3′; 5′-TGTGGTAGGCGATGATCAAA-3′; nested: 5′-GATAACCATGCCCACCAGAC-3′; 5′-ATTTCAGGGCCAACGAAAAC-3′;

rMyHC: first: 5′-CATCAATGCCAACCGCAG-3′; 5′-TCCCGAGCATCCATTTCTTC-3′; nested: 5′-AGGCCACTGAGAGCAATGAG-3′; 5'-TCAATAACTCTACGGCCTCCA-3′;

reNOS: first: 5′-GAGAGGCAGGCTGTCAATG-3′; 5′-GGGAGTCCTTCCTTCGAGTG-3′; nested: 5′-CCAGCTCTGTCCTCAGAAGG-3′; 5′-ATGGATGAGCCAACTCAAGG-3′.

GenBank™ accession numbers: rIKCa1: AF156554; rSlo: AF135265; rSK1: AF000973; rSK2: U69882; rSK3: U69884; rMyHC: X16262; reNOS: AJ011116; rGAPDH: AB017801.

Statistical analysis. Data are given as mean ± SE. If appropriate, the Wilcoxon Rank-Sum test or χ-square analyses were used to assess differences between groups. P-values of $P<0.05$ were considered significant.

Results

Alterations in K_{Ca} functional expression in neointimal VSMC following BCI. To measure functional K_{Ca} channel expression, we performed whole-cell patch-clamp experiments in combination with 'single-cell' RT-PCR analysis on neointimal VSMC *in situ* and on freshly isolated mature VSMC (12,13). Mature VSMC (n = 14) from normal CA exhibited an outward Ca^{2+}-activated and voltage-dependent K^+ current with characteristics of the cloned BK_{Ca} channel (4,5,12,13,14). The outward K^+ current was small at negative membrane potentials, increased steeply at depolarizing positive membrane potentials, and was blocked by the selective BK_{Ca} inhibitor, IbTX (Figure 1a, left panel), with a potency similar to the cloned BK_{Ca} channel (K_D 11± 3 nM, Fig. 1a, left inset). The selective SK_{Ca} blocker apamin (APA, 1 μM), and the IK_{Ca} blockers TRAM-34 (1 μM) and CLT (1 μM) (6,7,15) had no effect on this current (data not shown). The BK_{Ca} opener NS1619 stimulated the current, whereas 1-EBIO, an opener (16) of IK_{Ca} and SK_{Ca}, had no detectable effect (not shown). A small residual voltage-gated Ca^{2+}-independent (K_v) K^+ current (1.1 ± 0.2 pA/pF at 0 mV) in these cells was sensitive to 2 mM 4-aminopyridine (Figure 1a, right panel). The voltage-dependence of the composite BK_{Ca} plus K_v current in mature VSMC, normalized for cell capacitance (I_K [pA/pF]), is shown in Figure 1d.

Two weeks after BCI, neointimal VSMC (n = 30) exhibited a substantially altered K^+ current pattern. In a majority of neointimal VSMC (19 of 30), two calcium-activated K^+ currents were seen (Figure 1b, top left panel) with properties resembling BK_{Ca} and IK_{Ca} channels. The IK_{Ca} component seen at negative potentials was eliminated by the selective IK_{Ca}-inhibitor TRAM-34, leaving a residual BK_{Ca} current that increased steeply at positive potentials. A combination of TRAM-34 and IbTX completely suppressed both components (Figure 1b, top left panel). In 11 of 30 of these neointimal VSMC, BK_{Ca} currents were absent, and these cells contained only IK_{Ca} currents (Fig. 1b top right panel and both bottom panels). These currents

were half-maximally activated by ~350 nM $[Ca^{2+}]_I$ (Figure 1b bottom left panel), and were blocked by TRAM-34 (K_D 10 ± 2 nM), CLT (K_D 31 ± 4 nM) and charybdotoxin (ChTX; K_D 5 ± 1 nM, Figure 1b bottom right panel) with potencies similar to the cloned channel (18), but not by 1 μM of the SK inhibitor APA or 2 mM 4-aminopyridine (not shown). The IK_{Ca} opener 1-EBIO (100 μM, n = 7, not shown) increased the amplitude of the current by 202 ± 29%. These properties of the IK_{Ca} current in neointimal VSMC are remarkably similar to the cloned IKCa1 channel, and the native IK_{Ca} channel in human and rat endothelial cells (12,13), proliferating rat aortic VSMC (17), human lymphocytes (6,7,15), human pancreas (18), fibroblast cell lines (8). These results demonstrate a significant shift from predominantly BK_{Ca} functional expression in mature VSMC to a mixture of IK_{Ca} and BK_{Ca} in neointimal cells two weeks post BCI.

Six weeks after BCI, both types of K_{Ca} currents were missing or were so small that they were not clearly distinguishable from non-specific leak in the majority of neointimal VSMC (Figure 1c, n = 24; cell capacitance: 14 ± 1 pF). Only two cells exhibited IK_{Ca} currents and only one cell displayed BK_{Ca} currents. When normalized for membrane capacitance, a measure of cell size, the mean K^+ current six weeks after BCI was greatly reduced compared to mature VSMC or neointimal VSMC two weeks after BCI (Figure 1d).

Alterations in BK_{Ca} and IKCa1 mRNA expression in neointimal VSMC following BCI correlate with changes in functional expression. We used 'multiplex' single-cell RT-PCR to determine whether the changes in functional BK_{Ca} and IK_{Ca} expression following BCI were correlated with alterations in mRNA levels for the rSlo and IKCa1 genes, respectively. The VSMC marker rMyHC was detected in all mature VSMC (34/34), in all neointimal VSMC (18/18) two weeks post BCI, and in 63 % (19/30) of neointimal VSMC six weeks after BCI. Endothelial cell-specific eNOS expression was not detected in any of the cell samples, demonstrating that our VSMC

samples are not contaminated with endothelial cells. None of the negative controls (n = 24) yielded any PCR products.

Consistent with the electrophysiology data in Figure 1, mature VSMC that express BK_{Ca} and not IK_{Ca} channels contained substantial quantities of rSlo mRNA (87 %; 54/62) and no rIKCa1 mRNA (0/27; Figure 2a and b). Transcripts of the related SK1-SK3 genes were also not detected in these cells (Figure 2a). Two weeks following BCI, the K_{Ca} gene expression pattern in neointimal VSMC was altered (Figure 2a and b) in keeping with the changes observed in the amplitude of BK_{Ca} and IK_{Ca} currents in these cells (Figure 1). We detected rSlo transcripts significantly less frequently in these cells (24/67; 36 %) compared to mature VSMC ($P<0.01$, χ-square analysis), whereas rIKCa1 transcripts were more frequently detected (42/67; 63 %; $P<0.001$). Interestingly, a faint rSK3 band is detected in these neointimal VSMC (Figure 2a), although the contribution of SK3 to the K_{Ca} current must be small because it was insensitive to 1 μM APA and a combination of TRAM-34 and IbTX completely suppressed the current (Figure 1a, left). Corroborating the decreased functional expression of BK_{Ca} and IK_{Ca} currents in VSMC six weeks post BCI, rSlo was detectable in only 6 % (4/69) of these samples and rIKCa1 in only 16 % (11/69). These results indicate that changes in Slo and IKCa1 mRNA levels following BCI contribute to the observed changes in BK_{Ca} and IK_{Ca} functional expression in VSMC.

EGF-induced up-regulation of IKCa1 expression and proliferation of rat VSMC via MEK activation. Activation of the Ras/Raf/MEK/ERK-signaling system has been shown to up-regulate IK_{Ca} expression and thus affect proliferation of rat fibroblast *in vitro* (8). IK_{Ca} up-regulation in VSMC following BCI might therefore be mediated by activation of this signaling pathway. To test this hypothesis, we compared IK_{Ca} function and rIKCa1 expression in the aortic VSMC cell line A7r5 before and 48 hours after stimulation with the mitogenic factor EGF. Following

stimulation, the amplitude of the IK_{Ca} current increased threefold compared to untreated cells (Figure 3a, left panel; $P<0.01$). Both treated and untreated A7r5 cells were devoid of substantial BK_{Ca} or voltage-gated K^+ currents. The IK_{Ca} current was activated by Ca^{2+} with an EC_{50} of ~350 nM and was blocked by TRAM-34 (K_D 10 ± 1 nM, not shown) with a potency similar to IKCa1. Involvement of the MEK/ERK pathway in EGF-induced IK_{Ca} up-regulation was demonstrated with the MEK-inhibitor PD98059. Pretreatment with PD98059 (20 μM) for 30 min prior to EGF stimulation prevented the increase in IK_{Ca} current amplitude (Fig. 3a), similar to the FGF effect in fibroblasts (8). Parallel RT-PCR studies revealed a 3-fold increase in rIKCa1 transcript levels 48 hours following EGF stimulation, which was blocked by PD98059, but not by the p38-MAP kinase inhibitor SB203580 (Figure 3a, right panel); a 6-fold increase in IKCa1 mRNA levels was detected as early as 2 hours after EGF stimulation (Table 1). In contrast, thrombin (1 U/ml) was ineffective in augmenting IK_{Ca} current amplitude or in up-regulating expression of rIKCa1 transcripts (Table 1). Taken together, these results show that EGF-stimulated A7r5 cells resemble proliferating neointima *in vivo*, suggesting that EGF-induced activation may contribute to the increased IK_{Ca} expression seen in neointimal VSMC two weeks post BCI.

To test whether the enhanced IK_{Ca} expression in VSMC might have functional consequences, we examined whether the IKCa1 inhibitors TRAM-34 (1 μM) and CLT (1 μM) could suppress EGF-stimulated mitogenesis of A7r5 cells. EGF significantly induced mitogenesis of these cells, which was significantly greater than that observed in unstimulated or thrombin-stimulated cells (Figure 3c). TRAM-34 and CLT suppressed mitogenesis to the levels seen in unstimulated cells (Figure 3c). The MEK-inhibitor PD98059 completely suppressed EGF-induced mitogenesis, while the p38-MAP kinase inhibitor SB203580 had no effect (Figure 3c). These results suggest that the IK_{Ca} channel plays a role in neointimal proliferation as it has been reported to do in lymphocytes and fibroblasts (6,7,8).

TRAM-34 and CLT suppress BCI-induced intimal hyperplasia in vivo. Based on the up-regulation of IK_{Ca} channel expression in VSMC following BCI and the effectiveness of IK_{Ca} blockers in suppressing EGF-induced proliferation of A7r5 cells, we examined whether IK_{Ca} blockade might reduce intimal hyperplasia in the carotid arteries of rats following BCI. The data are summarized in Table 2 and representative cross-sections of CA of each group are shown in Figure 4.

An initial trial with CLT (120 mg/kg/d administered subcutaneous) for two weeks provided encouraging results, but the CLT-treated rats gained significantly less weight than the vehicle-treated group and developed hepatomegaly due to CLT's reported liver toxicity mediated via inhibition of P450-dependent enzymes (22). We therefore switched to the more selective IK_{Ca} inhibitor TRAM-34 (120 mg/kg/d, subcutaneous), which has no effect on P450-dependent enzymes (6) and should therefore not be liver toxic. In the vehicle-treated group neointima formation progressively increased from week-1 to week-6 post-BCI. We observed a progression of neointima formation in the TRAM-34-treated group, but the area of the neointimal-cell layer in these rats was significantly smaller than vehicle-treated rats at week-1 (-64 %; $P<0.01$), week-2 (-35 %; $P<0.01$), and week-6 (-43 %; $P<0.01$) post BCI (Figure 4 and Table 2). Two weeks treatment with CLT also resulted in a pronounced reduction of neointimal formation (-50 %; $P<0.001$, Figure 4 and Table 2). The area of the medial smooth-muscle-cell layer was not different between rats treated with TRAM-34, CLT, or vehicle. The ratio of neointimal/medial areas (N/M) in TRAM-34- and CLT-treated rats was therefore significantly smaller than that of the respective vehicle-treated groups at all times measured post-BCI. The reduced neointima formation in TRAM-34-treated animals resulted in significantly larger residual lumina at two weeks (+34 %; $P<0.05$) and at six weeks (+44 %; $P<0.01$) after BCI compared to vehicle-treated rats. Due to the low amount of neointima formation at week-1 post-BCI, there was no statistical difference in lumen area of TRAM-34-treated rats and vehicle-controls. CLT-treated animals also

displayed larger residual lumina at two weeks (+49 %; $P<0.001$) after BCI. We normalized the lumen area of the injured CA (rL) to that of the uninjured contralateral CA (rL/cL). Table 2 shows that TRAM-34-treated rats displayed a lower degree of lumen narrowing (higher rL/CL values) at week-2 (-9 %; $P<0.01$) and week-6 (-19 %; $P<0.01$) compared to vehicle-treated controls (-36 % at week-2 and -50 % week-6). A lower degree of lumen narrowing was also observed in the CLT-treated group at two weeks after BCI (-18 %; $P<0.05$).

TRAM-34 treatment caused no visible side effects or organ damage as determined macroscopically during the course of the study. After transient weight loss in the first week due to surgery, TRAM-34-treated rats gained weight (30 ± 5 g after two weeks; 99 ± 6 g after six weeks) similar to the vehicle-treated group (25 ± 4 g after two weeks, 90 ± 15 g after six weeks). In contrast, the CLT-treated group gained significantly less weight (7 ± 6 g; $P<0.05$) within two weeks after BCI. Subcutaneous injections of TRAM-34 and CLT resulted in serum levels of 102 ± 21 nM and 375 ± 75 nM, respectively, as determined with a bioassay at the end of the treatment and 24 h after the last injection.

To understand the mechanism by which TRAM-34 and CLT reduced neointima formation, we investigated cell proliferation, apoptosis, and extracellular matrix (collagen) content. The neointimal nuclei count, a measure of cell proliferation, was reduced by -70% ($P<0.05$) after one week, by -39% ($P<0.01$) after two weeks, and by -61% ($P<0.001$) after six weeks of TRAM-34-treatment compared to vehicle-treated rats. A similar reduction (-59%, $P<0.001$) in neointimal nuclei count was observed in the CLT-treated group at two weeks after BCI. However, the collagen content and the rate of apoptosis (percentage of apoptotic nuclei) in the neointima was not different in TRAM-34- and CLT-treated rats compared to vehicle-treated controls (Figure 5a and b). Taken together our results demonstrate that IK_{Ca} blockers reduce neoimtima formation by inhibition of VSMC proliferation.

Discussion

Percutaneous balloon angioplasty, a procedure used to relieve arterial stenosis and improve blood flow, is frequently complicated by vascular restenosis due to proliferation of VSMC. Using a balloon catheter injury model to the rat carotid artery, we demonstrate that neointimal formation following angioplasty is associated with an alteration in K_{Ca} channel expression in VSMC. Mature VSMC exclusively expressed BK_{Ca}, whereas proliferating neointimal cells down-regulated BK_{Ca} and up-regulated IKCa1. Blockade of IKCa1 inhibited EGF-induced proliferation of VSMC *in vitro* and reduced neointimal formation *in vivo* post-BCI. IKCa1 blockade might therefore represent a novel therapeutic strategy for the prevention of restenosis following angioplasty.

Neointimal proliferation and IKCa1 up-regulation following BCI is mediated by numerous mitogenic factors. Using the aortic VSMC cell line A7r5 as a model system we demonstrated that EGF augmented IKCa1 RNA and functional expression, and induced proliferation, via activation of the MEK/ERK signaling pathway. IKCa1 has been similarly reported to be up-regulated and to contribute to the proliferation of growth factor-stimulated fibroblasts (8) and mitogen-activated human T lymphocytes (6,7,15,23). In fibroblasts, like VSMC, IKCa1 up-regulation is mediated through the Ras/Raf/MEK/ERK signaling cascade, and in T-lymphocytes augmentation of IKCa1 levels occurs as a result of AP1-dependent transcription. Thrombin, another putative mitogen for VSMC, failed to up-regulate IKCa1 expression or induce mitogenesis, possibly because it acts more as a stimulus for differentiation rather than as a mitogenic factor in VSMC (11). Enhanced IKCa1 expression may therefore be a functional characteristic of proliferating and de-differentiated cells (8,17).

IKCa1 might promote VSMC mitogenesis by enhancing the electrochemical driving force for Ca^{2+} influx via membrane hyperpolarization and thus sustain a high intracellular Ca^{2+}

concentration, as has been reported in lymphocytes and fibroblasts (6,7,23,24). IKCa1 may play a more important role than BK_{Ca} in regulating the membrane potential and calcium signaling of proliferating VSMC because its higher Ca^{2+} affinity (3,4,6,7,12,14,15,17,18) would result in IKCa1 channel opening and membrane hyperpolarization in response to subtle increases in the intracellular Ca^{2+} concentration. Induction of IKCa1 expression might thus be an essential step in promoting neointimal VSMC proliferation following BCI. Consistent with such a role, IKCa1 blockade by CLT, ChTX and the specific inhibitor TRAM-34 suppressed the proliferation of cultured VSMC. IKCa1 blockers may therefore have therapeutic value for preventing neointimal proliferation and restenosis following BCI.

In a rat model of BCI, administration of CLT significantly reduced neointimal thickening, but the trial was discontinued after two weeks due to the development of severe hepatomegaly and reduced weight gain, presumably because of liver toxicity (22) caused by blockade of cytochrome P450-dependent enzymes (6). A subsequent trial with TRAM-34, an IKCa1 selective inhibitor that does not block cytochrome P450 enzymes (6), significantly reduced neointimal hyperplasia without causing visible signs of organ damage or gastrointestinal side-effects. TRAM-34's therapeutic effect was due to inhibition of neointimal cell proliferation and not due to increased apoptosis or decreased matrix formation. In conclusion, targeting IKCa1 channels in proliferating VSMC with TRAM-34 might have therapeutic utility in the prevention of restenosis after angioplasty, and for the treatment of other cardiovascular disorders characterized by abnormal VSMC proliferation.

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Table 1:

Mitogenic Regulation of rIKCa1 Expression and IK_{Ca} Function in Rat Aortic VSMC (A7r5)

Cell Treatment	n	GAPDH (Ct)	rIK1 (ΔCt)	rIK1 (ΔΔCt) (x-fold Increase)	rMyHC (ΔCt)	Cell Treatment	n (Cells)	I_{rIK1} (pA/pF)
w/o	16	22.6 ±0.5	12.4 ± 0.4		9.2 ± 0.3	w/o	14	0.8 ± 0.2
EGF (2 h)	17	22.7 ± 0.6	9.6 ± 0.4***	2.8 (~6-fold)		EGF	16	3.4 ± 0.6***
EGF (48 h)	11	21.7 ± 0.8	10.8 ± 0.4**	1.6 (~3-fold)	9.0 ± 0.4	EGF + PD98059	23	1.0 ± 0.2#
EGF + PD98059 (2 h)	2	22.9 ± 3.7	13.2 ± 0.2	-0.8		EGF + SB203580	7	2.2 ± 0.5**
EGF + PD98059 (48 h)	4	22.7 ± 0.4	12.6 ± 0.6#	-0.2	9.4 ± 0.4			
EGF + SB203580 (48 h)	5	19.1 ± 1.7	10.7 ± 0.4*	1.7 (3-fold)	9.6 ± 0.2	Thrombin	11	0.4 ± 0.1
Thrombin (48 h)	2	23.8 ± 0.5	12.2 ± 0.9	0.2				

Real-time RT-PCR analysis of rIKCa1 and rMyHC expression (left) and whole cell currents of IK_{Ca} at 0 mV (right) in A7r5 cells following EGF stimulation for 48 h. Values are given as mean ± SE; $Ct_X - Ct_{rGAPDH} = \Delta Ct$; $\Delta Ct_{w/o} - \Delta Ct_X = \Delta\Delta Ct$; $2^{\Delta\Delta Ct}$ = fold increase in expression, e.g. 1 ΔΔCt = 2-fold; 2 ΔΔCt = 4-fold; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. w/o; # $P<0.05$, ## $P<0.01$, vs. EGF-stimulated cells; Wilcoxon Rank-Sum test.

Table 2:

Effect of TRAM-34 and CLT on intimal hyperplasia after BCI

Treatment group	n	Neointimal Area (mm^2)	Medial Area (mm^2)	N/M	Residual Lumen Area (mm^2)	rL/cL	Nuclei Count (Cell No.)	Rate of Apoptosis (%)	Collagen Content (%)
Vehicle									
1 week	4	0.011 ± 0.010	0.084 ± 0.005	0.13 ± 0.01	0.22 ± 0.01	0.93 ± 0.04	159 ± 17		
2 weeks	11	0.097 ± 0.006	0.099 ± 0.003	1.05 ± 0.06	0.16 ± 0.01	0.64 ± 0.05	984 ± 82	0.9 ± 0.6	11 ± 2
6 weeks after BCI	5	0.169 ±0.008	0.091 ± 0.008	1.92 ± 0.22	0.13 ± 0.01	0.50 ± 0.02	1525 ± 79	<<1	19 ± 3
TRAM-34									
1 week	4	0.004 ± 0.001*	0.084 ± 0.004	0.05 ± 0.01**	0.21 ± 0.02	0.96 ± 0.05	47 ± 16*		
2 weeks	6	0.063 ± 0.005**	0.095 ± 0.002	0.66 ± 0.05**	0.21 ± 0.02*	0.91 ± 0.06**	601 ± 36**	1.0 ± 0.6	15 ± 2
6 weeks after BCI	5	0.096 ± 0.018**	0.082 ± 0.006	1.15 ± 0.18**	0.18 ± 0.01**	0.81 ± 0.06**	612 ± 97***	<<1	11 ± 4
CLT									
2 weeks after BCI	7	0.049 ± 0.010***	0.102 ± 0.004	0.49 ± 0.10***	0.24 ± 0.02**	0.82 ± 0.05*	407 ± 82***	0.6 ± 0.5	13 ± 1

N/M = ratio of neointimal/medial areas; rL/cL = residual Lumen/contralateral Lumen; Values are given as mean ± SE;* $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. vehicle, Wilcoxon Rank-Sum test.

Legends

Figure 1: Mature VSMC express BK_{Ca} currents while neointimal VSMC express IK_{Ca} at two weeks and no K_{Ca} currents at six weeks after BCI. **(a)** *Left panel:* BK_{Ca}-currents in mature VSMC are elicited through dialysis with 3 μM free Ca^{2+} and blocked by 100 nM IbTX. Inset: concentration-dependent blockade of BK_{Ca}-currents by IbTX (n = 4-5). *Right panel:* Voltage-gated K^+ currents in mature VSMC recorded with a Ca^{2+}_{free} pipette solution and blockade by 4-AP. **(b)** *Left upper panel:* Mixed BK_{Ca} and IK_{Ca} currents in neointimal VSMC at two weeks after BCI and blockade of IK_{Ca} currents by TRAM-34 and BK_{Ca} currents by IbTX. *Right upper panel:* Concentration-dependent blockade of IK_{Ca}-currents by TRAM-34 in cell expression pure IKCa1 current. *Lower panel from left to right:* Ca^{2+}-dependence of IK_{Ca}-currents and pharmacology of IK_{Ca}-currents; TRAM-34 (n = 6-7; λ), CLT (n = 3-5; ○), and ChTX (n = 3-4; □). **(c)** Lack of K_{Ca} currents in neointimal VSMC at six weeks after BCI. **(d)** Quantitative analysis of IK_{Ca} and BK_{Ca} currents in mature VSMC (■) and neointimal VSMC at two (○) and six weeks (Δ) at holding potentials of -40, 0, and +100 mV. Values are given as mean ± SE; * $P<0.05$, ** $P<0.01$, neointimal VSMC at two weeks vs. mature VSMC; # $P<0.05$, ## $P<0.01$ neointimal VSMC at six weeks vs. mature VSMC; † $P<0.05$, †† $P<0.01$ neointimal VSMC at six weeks vs. neointimal VSMC at two weeks; Wilcoxon Rank-Sum test.

Figure 2: 'Multiplex' single-cell RT-PCR analysis of single mature and neointimal VSMC. **(a)** Ethidium bromide-stained gels of RT-PCR products of K_{Ca} genes (upper panel) and rMyHC (lower panel) in single mature and neointimal VSMC at two weeks after BCI and negative controls: one -RT control, one medium sample, and H_2O-control. **(b)** Representative expression pattern of the K_{Ca} genes rSlo and rIKCa1 (*upper panel*) and rMyHC (*lower panel*) in mature VSMC and neointimal VSMC at two and six weeks after BCI. Columns: Quantitative analysis of

rSlo and rIKCa1 expression in mature VSMC (rats, n = 9) and neointimal VSMC at two weeks (rats, n = 5) and six weeks (rats, n = 6). Values are given as mean ± SE; ** $P<0.01$, Wilcoxon Rank-Sum test.

Figure 3: EGF up-regulates IKCa1 expression and induces proliferation of the VSMC cell line, A7r5. (**a**) Patch-clamp and RT-PCR analysis of rIKCa1 expression following EGF stimulation in A7r5 cells. Representative K_{Ca} currents (*left*) in unstimulated (w/o) and EGF-stimulated cells (48h) in the absence or presence of the MEK-inhibitor PD98059. Ethidium bromide-stained gel (*right*) of real-time RT-PCR products of rIKCa1 in unstimulated and EGF-stimulated cells with or without PD98059 (20 μM) or the p38-MAP kinase inhibitor SB203580 (5 μM). (**b**) EGF, but not thrombin, induces proliferation of A7r5 cells, which is suppressed by PD98059 (20 μM), TRAM-34 (1 μM) and CLT (1 μM), but not SB203580 (5 μM). The % increase in cell number is shown. Cell counts were made 48 hours after stimulation. Conditions: thrombin (n = 6), EGF (n = 13), EGF + PD98059 (n = 7), EGF + SB203580 (n = 5), EGF + TRAM-34 34 (n = 6), EGF + CLT (n = 6). Values are given as mean ± SE; * $P<0.01$ vs. w/o, # $P<0.001$ vs. EGF, Wilcoxon Rank-Sum test.

Figure 4: TRAM-34 and CLT reduce neointima formation following BCI. *Upper panel:* Representative cross-sections of carotid arteries stained with hematoxylin & eosin after treatment with TRAM-34 or vehicle at one week after BCI; original magnification 200x; Arrows indicate neointima/media borders. *Middle panel:* Representative cross-sections after treatment with TRAM-34, CLT, or vehicle at two weeks after BCI; original magnification 50x. *Lower panel:* Representative cross-sections of carotid arteries after treatment with TRAM-34, CLT, or vehicle at six weeks after BCI; original magnification 50x.

Figure 5: TRAM-34 and CLT had no effect on collagen content or rate of apoptosis in the neointima following BCI. **(a)** Representative cross-sections stained with Sirius Red (collagen stain) after treatment with TRAM-34, CLT, or vehicle at two weeks after BCI; original magnification 100x. **(c)** Representative cross-sections stained by use of the TUNEL method for detection of apoptotic nuclei in injured carotid arteries after treatment with TRAM-34, CLT, or vehicle at two weeks after BCI; sections were counterstained with methyl green to visualize all nuclei; original magnification 400x; arrows indicate apoptotic nuclei in the neointima.